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FERTILITY PRESERVATION

Cryo-survival, fertilization and early embryonic development of vitrified oocytes derived from mice of different reproductive age

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Abstract
Purpose To evaluate the effect of female reproductive age on oocyte cryo-survival, fertilization and the subsequent embryonic development following vitrification using the mouse model in order to address the question of how maternal reproductive age is related to fertility preservation.
Methods Oocytes were collected from mice of different reproductive age: (1) 8–10 weeks, (2) 16–20 weeks, (3) 32–36 weeks, and (4) 44–48 weeks. Following vitrification and warming, the oocytes in each group were assessed for cryo-survival, fertilization and embryonic development as well as for the quality of blastocysts. Fresh oocytes without undergoing vitrification were used in each age group as controls.

Capsule Reduction in fertilization rate and poor embryonic development following vitrification procedure has been found to be associated with increased reproductive age.

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Results The mean number of oocytes retrieved following superovulation was found to reduce significantly ($P<0.05$) in mice from 32–36 weeks of age (18.1±8.5) compared with 8–10 weeks of age (26.8±9.8) and 16–20 weeks of age (23.9±4.2) respectively. The cryo-survival rate of oocytes was reduced significantly ($P<0.05$) in mice of 44–48 weeks of age (90.4%±7.9) compared with the other 3 groups (98.8%±2.1, 98.0%±3.3 and 98.5%±2.2, respectively). The cleavage rate of vitrified oocytes declined significantly following the increase in maternal age in mice of 32–36 weeks of age (69.7%±20.8) forward (63.6%±9.2). However, no significant difference in the cleavage rate was found among the control groups of different maternal ages. The rate of embryo development to the blastocyst stage in the vitrified oocytes also significantly declined following the increase in maternal age (71.8%±8.8, 66.4%±10.7, 64.2%±17.4 and 4.1%±8.3 respectively). There were no such differences in the rates of embryo development to the blastocyst stage among the control groups following the increase in maternal age (75.9%±12.2, 79.5%±28.9, 70.2%±17.4 and 69.3%±19.0 respectively). However, the quality of blastocysts produced from 32–36 weeks and 44–48 weeks of ages was significantly poor in term of total cell numbers and the ratio of inner cell mass(ICM) / trophectoderm (TE) compared to younger age in both vitrified and control groups
Conclusions Cryo-survival of oocytes following vitrification and warming procedures is associated with female reproductive age. There is a more negative impact on the oocytes following vitrification and warming with the increase of maternal age.

Keywords Female reproductive age · Oocytes · Cryo-survival · Fertilization · Embryonic development · Blastocyst

Introduction

In women, age-related decline of female fertility starts at the age of 30 s, markedly accelerates after 35 years of age, and fertility loss starts at a mean age of 41 years and completes by 50 years of age [1–3]. Studies on "natural populations" artificial insemination programs with donor sperm and in vitro fertilization (IVF) programs with women's own oocytes have provided strong evidence for fertility decline during the third and fourth decades of life [4–6]. Although the primary cause for this decline is the gradual depletion of oocytes in aging ovary, it has been suggested that the decline of oocyte quality is also an important contributing factor [7]. Studies of older women (>40 years of age) undergoing IVF of donation with young oocytes indicated that oocyte quality decline is the major cause for infertility in older women, and oocyte age can even compensate for other age-related changes in the endocrine system and reproductive tract [8, 9]. Therefore, it appears that the quality of oocytes declines with maternal aging and affects embryonic development [3, 10, 11].

With the improvement of oocyte cryopreservation technologies, it is now possible to preserve female fertility having the oocyte cryopreserved prior to cancer treatment with cytotoxic chemotherapy or radiotherapy [12]. Oocyte cryopreservation would be a better option for women without a male partner. Recent experimental evidence indicated that, with the advancement made in oocyte vitrification technology, the use of cryopreserved oocytes have resulted in more and more healthy live births [13–15]. Although oocyte cryopreservation is still considered an experimental procedure by the American Society for Reproductive Medicine [16, 17], today cancer patients, who are undergoing invasive cancer treatments and who wish to preserve their oocytes, are increasingly considering oocyte vitrification as a potential option for cryopreservation of their oocytes.

In addition, the idea of using Assisted Reproductive Technologies (ART) to conceive a child is appealing only to a minority of single women [18]. With the increasing opportunities for higher education, career advancement and economic independence, combined with the availability of highly effective contraception methods, more young women are delaying childbearing until the fourth decade of life regardless of the potential fertility decline [1, 19]. As a result, more and more women consider oocyte cryopreservation as a means to preserve their fertility even when they are already at the fourth decade of age. Apart from the association of declining quality of oocytes with maternal aging which also affects embryonic development, the effect of maternal age on cryo-survival, fertilization and embryonic development of vitrified oocytes is still largely unknown.

The objective of this study was to use the mouse model to examine the effect of female reproductive age on oocyte cryo-survival, fertilization and the subsequent embryonic development following vitrification in order to address the question of whether maternal reproductive age affects fertility preservation.

Materials and methods

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless specified otherwise.

Oocyte collection and sperm preparation

The Animal Care Committee of McGill University approved all experiments involving the use of animals in this study. All mice (CD-1) were kept under specific-pathogen-free conditions with a humidity range of 30–60%, a temperature range of 21–24°C, a light cycle of 12 h light:12 h darkness, and were given free access to sterile food and water.

Female CD-1 mice were superovulated with an intraperitoneal injection of 10 IU of pregnant mare's serum gonadotropin (PMSG), 48 h later followed by an intraperitoneal injection of 10 IU of human chorionic gonadotropin (HCG). Cumulus-oocytes-complexes (COCs) were collected from the ampullar region of oviducts 14 h after HCG injection. Cumulus cells were dispersed by 80 unit/mL of hyaluronidase for 1 min and removed by gently pipetting, and the denuded metaphase-II (M-II) oocytes were used for the experiments.

To collect sperm from the cauda epididymis, male CD-1 mice (10–14 weeks of age) were sacrificed by cervical dislocation, and both epididymides were dissected from the testes in their entirety. The contents in each cauda epididymis were squeezed out with a pair of forceps and transferred immediately into a pre-warmed drop (0.4 mL) of modified human tubal fluid (mHTF) containing 0.9% bovine serum albumin (BSA) for a 90-min incubation at 37°C in 5% CO_2 in humidified air to induce sperm capacitation.

Oocyte vitrification and warming procedures

The vitrification and warming procedures were carried out as previously described [20]. Briefly, the oocytes were suspended for 3 min in an equilibration solution containing 7.5% ethylene glycol (EG), 7.5% 1.2-propanediol (PROH) and 10% fetal bovine serum (FBS) in Dulbecco's phosphate buffered saline (DPBS). Afterwards the oocytes were transferred to vitrification solution (containing 15% EG,

15% PROH, 0.5 M sucrose and 10% FBS in DPBS) for 45–60 sec at room temperature, 4–5 oocytes were then loaded onto McGill Cryoleaf (MediCult Company, Denmark) and immediately plunged into liquid nitrogen for storage. For warming, the frozen McGill Cryoleaf carrying the oocytes was inserted directly into a thawing solution (containing 1.0 M sucrose in 10% FBS-supplemented DPBS) at 37°C for 1 min. The thawed oocytes were transferred to 0.5 M and 0.25 M sucrose in 10% FBS-supplemented DPBS for 3 min, respectively, and then washed twice with washing medium (10% FBS in DPBS) before they were transferred to culture medium at 37°C in 5% CO_2 in humidified air. Cryo-survival rate of the oocytes was assessed 2 h after incubation, and cryo-survived oocytes were characterized by the morphological appearance of membrane integrity and discoloration of the ooplasm. The surviving oocytes were used further for the subsequent experiments.

Insemination with intracytoplasmic sperm injection (ICSI) and embryo culture in vitro

ICSI was performed using an Olympus microscope equipped with Narishigue micromanipulators and Piezo system (Prime Tech, Japan). After sperm capacitation, 5 μL of sperm suspension were added to a droplet consisted of 5 μL of 12% PVP solution under paraffin oil previously prepared in a 60-mm petri dish (FALCON, USA). Prior to ICSI, oocytes were transferred from mHTF medium into a droplet of Hepes buffered mHTF medium under paraffin oil. With the sperm/PVP suspension in the same dish, only highly motile sperm with a morphologically normal head were selected, and the head was separated from the tail by applying a few Piezo pulses. The sperm head was injected into the oocyte using a Piezo drive unit [21]. After injection, the oocytes were washed thoroughly with mHTF medium and then transferred into a droplet containing 50 μL of embryo maintenance medium (SAGE Media, USA) and cultured in the medium for 120–122 h under paraffin oil at 37°C in 5% CO_2 in humidified air.

Assessment of the quality of blastocysts with differential staining

The number of inner cell mass (ICM) and trophectoderm (TE) cells in the blastocysts was determined with the method developed by Handyside and Hunter [22] with modifications. Briefly, the zona pellucida was removed from the blastocysts by culturing the latter in mHTF containing 0.5% protease for 15 min at 37°C in 5% CO_2 in humidified air. After rinsing with 0.5% PVP/PBS for 5 min, the naked blastocysts were transferred to a solution containing rabbit anti-mouse splenocyte antiserum and Hepes-mHTF (1:3) for 20 min at 37°C in 5% CO_2 in humidified air. The blastocysts were then washed 3 times each for 15 min with 0.5% PVP/PBS, and then immersed in a solution containing guinea pig complement and Hepes-mHTF (1:5) for another 20 min at 37°C in 5% CO_2 in humidified air. After rinsing with 0.5% PVP/PBS, the blastocysts were incubated for 15 min at room temperature with a staining solution containing Hoechst 33342 (5 μg/mL) and Propidium iodide (5 μg/mL) in 0.5% PVP/PBS. The stained blastocysts were mounted on microscope slides with mounting medium. Following mounting, the number of ICM and TE cells was counted under UV light with blue filter. The nuclei in the TE cells showed a red-pink color while the nuclei in the ICM cells displayed a bluish color.

Experimental design

COCs were collected from mice of four different reproductive age groups: (1) 8–10 weeks, (2) 16–20 weeks, (3) 32–36 weeks, and (4) 44–48 weeks. Each experiment was repeated 5 times.

Experiment 1: Effect of reproductive age on the ovary in response to gonadotropin stimulation. Following stimulation with PMSG and HCG injection, the mean number of oocytes from each mouse was compared in each age group;

Experiment 2: Cryo-survival rate of the vitrified oocytes from different reproductive age. Following vitrification, the oocytes were stored in liquid nitrogen for at least 7 days. After thawing, the mean number of survived oocytes was compared in each age group;

Experiment 3: Fertilization and embryonic development of the vitrified oocytes derived from mice of different reproductive age. Following ICSI, fertilization (2-cell cleavage) rate was assessed 16–18 h after ICSI, and embryonic development to 8-cell stage was observed 70–72 h after ICSI. Blastocyst development was assayed 120–122 h after ICSI. Fresh oocytes without cryopreservation from each matched age group were collected and inseminated by ICSI as controls

Experiment 4: Quality of blastocysts produced by vitrified oocytes derived from mice of different reproductive age. The quality of blastocysts was evaluated by differential staining method following fixation at 96–98 h after ICSI. Fresh oocytes without cryopreservation from each matched age group were collected and inseminated by ICSI as controls.

Table 1 Effect of mouse reproductive age on the ovary in response to gonadotropin stimulation (5 replicates)

Mouse age (weeks)	No. of mice examined	No. of oocytes retrieved	No. (mean) of oocytes from each mouse	95% confidence interval (CI)
8–10	18	483	26.8±9.8[a]	21.9~31.7
16–20	14	335	23.9±4.3[a]	21.4~26.4
32–36	17	309	18.1±8.5[b]	13.7~22.5
44–48	27	256	8.7±4.0[c]	4.5~10.1

[a–c] Different superscripts indicate significant difference ($P<0.05$)

Statistical analysis

The difference in the number of oocytes collected, the total cell number of blastocysts and the ratio of ICM/TE in each group were analyzed by ANOVA and a Fisher protected least significant difference test. The difference in oocyte cryo-survival, fertilization and embryonic development rates were analyzed by Chi-Square test, and quality control between each replicate were analyzed by ANOVA shown as mean±SD for different rates. The SPSS 17.0 statistical software package was employed in this analysis. Differences at $P<0.05$ (two-tailed) were considered to be statistically significant.

Results

As shown in Table 1, the mean number of oocytes retrieved following superovulation was found to decrease significantly ($P<0.05$) in mice of 32–36 weeks of age (18.1±8.5) compared with the groups of 8–10 weeks of age (26.8±9.8) and 16–20 weeks of age (23.9±4.2). The mean number of oocytes (8.7±4.0) collected from mice in the 44–48 weeks age group was found to be significantly lower than that of the other 3 groups.

Cryo-survival rate of the oocytes was reduced significantly ($P<0.05$) in mice of the 44–48 weeks age group (90.4%±7.9) compared with the other 3 groups (98.8%±2.1, 98.0%±3.3 and 98.5%±2.2, respectively) (Table 2). However, there were no differences found in the oocyte cryo-survival rate in mice of the 8–10, 16–20 and 32–36 weeks age groups.

As shown in Table 3, the cleavage rate of the vitrified oocytes declined significantly following the increase in maternal age starting from mice of 32–36 weeks of age (69.7%±20.8) to mice of 44–48 weeks of age (63.6%±9.2) compared with the mice in the 8–10 weeks (90.7%±13.7) and 16–20 weeks (91.9%±10.5) age groups and the control oocytes without vitrification (91.5%±6.9, 94.2%±2.0, 91.9%±5.8 and 89.7%±5.1, respectively). Similarly, the rate of embryos developed from the vitrified oocytes to 8-cell stage decreased significantly in mice in the 16–20 weeks age group (70.0%±4.8) with a further decrease in the 32–36 weeks (70.2%±15.6) and 44–48 weeks age groups (8.3%±16.6) compared with the mice of 8–10 weeks old (87.8%±10.3) and the corresponding groups of control oocytes without vitrification (88.8%±5.8, 85.5%±18.7, 80.6%±12.3 and 81.0±11.6, respectively). Finally, the rate of embryo development from vitrified oocytes to the blastocyst stage declined significantly following the increase in maternal age of the animals (71.8%±8.8, 66.4%±10.7, 64.2%±17.4 and 4.1%±8.3, respectively). However, there were no differences found in the rate of embryo development in the control groups of oocytes without undergoing prior vitrification (75.9%±12.2, 79.5%±28.9, 70.2%±17.4 and 69.3%±19.0, respectively).

Table 4 shows the quality of blastocysts produced by oocytes derived from mice of different reproductive age. The total cell numbers in each blastocyt decreased significantly in those derived from vitrified oocytes of 32–36 weeks of age (59.8±29.3) and was found to be further reduced in the oocytes in the 44–48 weeks age group (43.2±37.3) compared with the vitrified oocytes in the 8–10 weeks (94.2±41.3) and 16–20 weeks (85.8±46.0) age groups. However, no significant differences were found in the total cell number of blastocysts between the vitrified oocytes and the control oocytes regardless of the age group. Although there were no differences in the ratio of ICM/TE in the blastocysts between the vitrified oocytes and the control oocytes

Table 2 Cryo-survival rate of vitrified oocytes from mice of different reproductive age (5 replicates)

Mouse age (weeks)	No. of oocytes vitrified	No. of oocytes survived	Survival rate (mean%±SD)	95% confidence interval (CI)
8–10	300	296	98.8±2.1[a]	97.6~100.1
16–20	220	217	98.0±3.3[a]	95.8~100.3
32–36	198	195	98.5±2.2[a]	67.1~100.2
44–48	144	133	90.4±7.9[b]	83.1~97.8

[a–b] Different superscripts indicate significant difference ($P<0.05$)

Table 3 Fertilization and embryonic development of vitrified oocytes from mice of different reproductive age (5 replicates)

Mouse age (wks)	Oocytes with(+) or without (−) vitrification	No. of oocytes inseminated	No. of oocytes cleaved (%)	No. of embryos developed to 8-cell stage (mean%±SD)	No. of embryos developed to blastocyst stage (mean%±SD)
8–10	+	71	62 (90.7±13.7)[a]	53 (87.8±10.3)[a]	44 (71.8±8.8)[a]
	−	72	68 (91.5±6.9)[a]	59 (88.8±5.8)[a]	48 (75.9±12.2)[a]
16–20	+	68	64 (91.9±10.5)[a]	43 (70.0±4.8)[b]	38 (66.4±10.7)[ac]
	−	72	68 (94.2±2.0)[a]	56 (85.5±18.7)[a]	51 (79.5±28.9)[a]
32–36	+	62	38 (69.7±20.8)[b]	29 (70.2±15.6)[b]	27 (64.2±17.4)[bc]
	−	86	80 (91.9±5.8)[a]	63 (80.6±12.3)[a]	54 (70.2±17.4)[a]
44–48	+	60	41 (63.6±9.2)[b]	8 (8.3±16.6)[c]	4 (4.1±8.3)[d]
	−	97	87 (89.7±5.1)[a]	68 (81.0±11.6)[a]	56 (69.3±19.0)[a]

[a–d] Different superscripts indicate significant difference within the same column ($P<0.05$)

from the 8–10 weeks (17.3%±16.2 vs. 22.6%±18.4) and 16–20 weeks (20.9%±12.3 vs. 23.9%±14.3) of age, the ratio of ICM/TE was significantly reduced in the vitrified oocytes in mice of 32–36 weeks (8.7%±9.9) and 44–48 weeks (9.1%±9.7) of age compared to the control oocyte groups (22.8%±12.8 and 24.2%±2.9, respectively).

Discussion

The reproductive life span of female mice is strain specific. It usually starts at the age of 6 weeks post-natal and lasts up to 12–14 months [23]. This study revealed that adult mouse ovarian response to gonadotropin stimulation declines with increasing reproductive age as shown by the decrease in the number of oocytes collected (Table 1). Our observation support the findings of a previous study showing that the number of oocytes retrieved declined with increased mouse maternal age [24]. The mechanism of poor ovarian response to gonadotropin stimulation is not clear at the present time. However, it is reasonable to believe that the deletion of ovarian reservation is the main cause for the fertility decline. It has been reported that the number of primordial and growing follicles in the mouse ovary is nearly exhausted by 13–14 months [25–27].

Although several previous studies reported that the quality of oocytes is associated with maternal age [10, 28–32], prior to the present study there has not been any report indicating whether cryo-survival of oocytes is related to maternal age of the oocyte. Ultrastructural abnormalities have been found in correlation with increase in maternal reproductive age of mouse oocytes [24]. Results of the present study demonstrated for the first time that cryo-survival of oocytes is related to maternal age, revealing that the cryo-survival rate of mouse oocytes significantly declined in oocytes retrieved from mice of 40–44 weeks of age (Table 2). The reproductive life span of female mice is strain specific, but it usually lasts up to 12–14 months [23]. Mice over 40 weeks of age are already near the end of their reproductive life span, and they bear smaller litters and have less implantation sites than younger females. Therefore, it is important to note that the maternal reproductive age is associated with not only the poor quality of the oocytes but also with cryo-survival following vitrification. It appears that oocytes derived from mice of advanced maternal reproductive age are more sensitive to extracellular stress compared to oocytes derived from younger females.

No significant differences were found in the cleavage rate and embryonic development of oocytes derived from mice of different reproductive age without vitrification

Table 4 Quality of blastocysts produced by vitrified oocytes from mice of different reproductive age

Mouse age (wks)	Oocytes with (+) or without (−) vitrification	No. of blastocysts examined	Total cell numbers in each blastocyst (mean±SD)	Ratio of ICM/TE (mean%±SD)
8–10	+	22	94.2±41.3[a]	17.3±16.2[a]
	−	20	99.1±44.2[a]	22.6±18.4[a]
16–20	+	22	85.8±46.0[a]	20.9±12.3[a]
	−	25	85.9±38.8[a]	23.9±14.3[a]
32–36	+	24	59.8±29.3[b]	8.7±9.9[b]
	−	21	61.7±37.2[b]	22.8±12.8[a]
44–48	+	4	43.2±37.3[c]	9.1±9.7[b]
	−	30	46.3±17.6[c]	24.2±2.9[a]

[a–c] Different superscripts indicate significant difference within the same column ($P<0.05$)

(Table 3). Our findings are in line with the hypothesis put forth in previous reports [5, 33]. However, the present study demonstrated that the rate of cleavage was significantly reduced in vitrified oocytes of the 32–36 weeks age group, and embryonic development (8-cell stage and blastocyst stage) was significantly reduced in vitrified oocytes of the 16–20 weeks age group, indicating that the vitrification and warming procedures have an adverse effect on oocyte fertilization and embryonic development. Interestingly the present results indicated that the detrimental affects seem to be directly related to the maternal reproductive age of the vitrified oocytes, suggesting that vitrification and warming procedures may have caused some damages to the oocytes and that the damages are likely associated with the advanced maternal age.

Oocyte quality can also be assessed by the total cell number of blastocysts and the ratio of ICM/TE [34]. Results of the present study indicated that although the total cell number of blastocysts is related to the maternal age of the oocytes rather than the vitrification and warming procedures, the ratio of ICM/TE in blastocysts is related not only to the maternal reproductive age but also to the vitrification and warming procedures (Table 4). It has been known that DNA fragmentation is significantly higher in oocytes of aged mice compared with younger adult mice [35]. This DNA damage can be exacerbated by cryopreservation [36, 37]. A significant DNA fragmentation has been found in vitrified bovine oocytes [38]. It has been reported that the process of oocyte vitrification can induce profound modifications to mitochondria, cortical granules, microvilli, oolemma, smooth endoplasmic reticulum (SER) and cytoskeleton [39–43]. Therefore, taken together, it appears that the quality of blastocysts is detrimentally affected by both maternal reproductive age and vitrification/ warming procedures.

There is a paucity of information about the effect of maternal reproductive age on the pregnancy outcome of vitrified oocytes. It has been reported that in humans with increasing maternal reproductive age, a decrease of mitochondria in the ooplasmic fraction, dilation of SER and Golgi complex, and an increase in vacuolarization occurs in the oocytes [44]. Although it has been reported that a woman of 40 years of age gave live birth successfully following cryopreservation of her own oocytes [45], it should be taken into consideration that the quality of the oocytes are related not only to the maternal reproductive age but also to the vitrification and warming procedures. Both of the advanced reproductive age and the vitrification and warming procedures will synergistically affect the oocyte cryo-survival, fertilization and embryonic development. Therefore, it is important to emphasize here that the age limitation of women should be considered before utilizing cryopreservation of oocytes for different purposes in assisted reproductive technology.

Conclusions

The present study has demonstrated for the first time that oocyte cryo-survival following vitrification and warming procedures is directly related to the female reproductive age. Oocytes derived from mice of advanced maternal reproductive age are more sensitive to extracellular stress when compared to oocytes derived from younger females, resulting in reduced cryo-survival, fertilization and embryonic development.

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